

## Additional *Potamogeton* Hybrids from China: Evidence from a Comparison of Plastid *trnT*–*trnF* and Nuclear ITS Phylogenies

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Hybridization is a common phenomenon in many plant genera, and among them is the world's largest aquatic genus, *Potamogeton* (Potamogetonaceae) with about 69 species and 50 hybrids reported. Here we provide genetic insights into *Potamogeton* hybrids from China, where ten or more putative hybrids exist, of which eight have been genetically confirmed. In the study presented here, we verified the hybrid status and inferred the origins of three *Potamogeton* hybrids using molecular phylogenetic analyses of plastid (chloroplast) *trnT*–*trnF* and nuclear ITS sequence data sets that include previously published and newly generated data. The hybrids identified were: 1) *P. ×inbaensis*, a known hybrid from Japan; 2) a hybrid between *P. perfoliatus* s.l. and *P. wrightii*, and 3) a hybrid between tetraploid maternal *P. distinctus* and diploid paternal *P. octandrus*. *Potamogeton ×inbaensis* is reported from China for the first time, whereas the latter two hybrids are new to science.

Key words: aquatic plants, hybridization, ITS, *Potamogeton*, *trnT*–*trnF*

Hybridization and polyploidy are important drivers of diversification and speciation in plants (Stebbins 1950, Grant 1971, Abbott 1992, Rieseberg & Wendel 2004, Soltis & Soltis 2009). These evolutionary processes are also known to be significant factors in the evolution of the world's largest aquatic genus, *Potamogeton* L. (Potamogetonaceae; ca. 69 species; Hollingsworth *et al.* 1998; Wiegand & Kaplan 1998). Molecular phylogenetic and karyological data indicate that relatively few polyploidization events occurred early in the evolutionary history of the genus (Lindqvist *et al.* 2006). In contrast, hybridization events seem to have been more numerous, with about 50 hybrids reported, based on traditional morphology-based investigations (Wiegand & Kaplan 1998). So far, molecular phylogenetic techniques have been used to confirm the hybrid status and origins of 30 of these (Table 1). In addition to dis-

playing morphological intermediacy relative to their presumed parental species, divergent sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA have been detected in homoploid hybrid plants (see references in Table 1). However, ITS polymorphism are apparently absent in polyploids, regardless of ploidy level. Given that the inferred polyploidization events in *Potamogeton* occurred relatively early in the evolutionary history of the genus, it is likely that heterogeneous ITS sequences were eliminated in polyploid lineages through concerted evolution.

Wang *et al.* (2007) studied Chinese *Potamogeton* hybrids using molecular phylogenetic analyses of ITS data. Although they concluded on the basis of the presence of ITS polymorphism that two of the taxa in their studies are putative hybrids (*P. hubeiensis* W. X. Wang and *P. intortusifolius* J. B. He, L. Y. Zhou & H. Q. Wang), they failed to note that three additional accessions in their data set, *P. lucens* L., *P. natans* L., and

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TABLE 1. An account of genetically confirmed *Potamogeton* hybrids.

Hybrid	Maternal	Paternal	Reference
<i>P. ×absconditus</i> *	<i>P. perfoliatus</i> *	<i>P. richardsonii</i> *	Kaplan <i>et al.</i> 2009*
<i>P. ×aemulans</i>	<i>P. bicupulatus</i>	<i>P. epihydrus</i>	Kaplan <i>et al.</i> 2009
<i>P. ×anguillanus</i>	<i>P. perfoliatus</i>	<i>P. wrightii</i>	Iida & Kadono 2002*, Du <i>et al.</i> 2010a
	<i>P. wrightii</i>	<i>P. perfoliatus</i>	Du <i>et al.</i> 2010a
<i>P. ×assidens</i>	<i>P. nodosus</i>	<i>P. perfoliatus</i>	Zalewska-Gałosz <i>et al.</i> 2010
<i>P. ×bottnicus</i>	<i>P. vaginatus</i>	<i>P. pectinatus</i>	King <i>et al.</i> 2001
<i>P. ×cooperi</i>	<i>P. crispus</i>	<i>P. perfoliatus</i>	Kaplan & Fehrer 2004
<i>P. ×exilis</i>	<i>P. natans</i>	<i>P. alpinus</i>	Kaplan & Fehrer 2011, Kaplan & Uotila 2011**
<i>P. ×fluitans</i>	<i>P. lucens</i>	<i>P. natans</i>	Fant <i>et al.</i> 2001a, Fant <i>et al.</i> 2005
	<i>P. natans</i>	<i>P. lucens</i>	Fant <i>et al.</i> 2001a, Fant <i>et al.</i> 2005
<i>P. ×hubeiensis</i>	<i>P. cristatus</i>	<i>P. octandrus</i>	Wang <i>et al.</i> 2007*, Du <i>et al.</i> 2010b
<i>P. ×inbaensis</i>	<i>P. wrightii</i>	<i>P. lucens</i>	Ito <i>et al.</i> 2007
	<i>P. lucens</i>	<i>P. wrightii</i>	Amano <i>et al.</i> 2008
<i>P. ×intortusifolius</i>	<i>P. perfoliatus</i>	<i>P. wrightii</i>	Wang <i>et al.</i> 2007, Du <i>et al.</i> 2009
	<i>P. wrightii</i>	<i>P. perfoliatus</i>	Wang <i>et al.</i> 2007, Du <i>et al.</i> 2009
<i>P. ×jacobsii</i>	<i>P. crispus</i>	<i>P. ochreateus</i>	Kaplan <i>et al.</i> 2011
<i>P. ×lanceolatifolius</i>	<i>P. nodosus</i>	<i>P. gramineus</i>	Kaplan & Fehrer 2011
	<i>P. gramineus</i>	<i>P. nodosus</i>	Kaplan & Fehrer 2011
<i>P. ×maemetsiae</i> ***	<i>P. friesii</i>	<i>P. ritulus</i>	Zalewska-Gałosz & Ronikier 2011
<i>P. ×malainoides</i>	<i>P. distinctus</i>	<i>P. wrightii</i>	Du <i>et al.</i> 2010a
<i>P. ×mirabilis</i>	<i>P. oakesianus</i>	<i>P. gramineus</i>	Kaplan <i>et al.</i> 2009
<i>P. ×nitens</i>	<i>P. gramineus</i>	<i>P. perfoliatus</i>	Kaplan & Fehrer 2006, Kaplan <i>et al.</i> 2009
<i>P. ×orientalis</i>	<i>P. pusillus</i>	<i>P. oxyphyllus</i>	Du <i>et al.</i> 2010b
<i>P. ×salicifolius</i> *	<i>P. lucens</i> *	<i>P. perfoliatus</i> *	Fant & Preston 2004*, Kaplan 2007*
<i>P. ×schreberi</i>	<i>P. nodosus</i>	<i>P. nanans</i>	Hollingsworth <i>et al.</i> 1995*, Kaplan & Fehrer 2009
<i>P. ×subobsutus</i>	<i>P. nodosus</i>	<i>P. alpinus</i>	Zalewska-Gałosz <i>et al.</i> 2009
<i>P. ×sudermanicus</i>	<i>P. acutifolius</i>	<i>P. berchtoldii</i>	Fant <i>et al.</i> 2001b, Fant <i>et al.</i> 2003
	<i>P. berchtoldii</i>	<i>P. acutifolius</i>	Fant <i>et al.</i> 2001b, Fant <i>et al.</i> 2003
<i>P. ×suecicus</i>	<i>P. pectinatus</i>	<i>P. filiformis</i>	Hollingsworth <i>et al.</i> 1996*, King <i>et al.</i> 2001
	<i>P. filiformis</i>	<i>P. pectinatus</i>	Hollingsworth <i>et al.</i> 1996*, King <i>et al.</i> 2001
<i>P. ×vepsicus</i>	<i>P. natans</i>	<i>P. praelongus</i>	Kaplan & Fehrer 2011
<i>P. ×versicolor</i>	<i>P. epihydrus</i>	<i>P. perfoliatus</i>	Kaplan <i>et al.</i> 2009
<i>Potamogeton</i> hybrid	<i>P. pusillus</i>	<i>P. foliosus</i>	Whittall <i>et al.</i> 2004, Les <i>et al.</i> 2009*
<i>Potamogeton</i> hybrid	<i>P. berchtoldii</i>	<i>P. vaseyi</i>	Les <i>et al.</i> 2009
<i>Potamogeton</i> hybrid	<i>P. nodosus</i>	<i>P. wrightii</i>	Du <i>et al.</i> 2010a
<i>Potamogeton</i> hybrid	<i>P. distinctus</i>	<i>P. gramineus</i>	Du <i>et al.</i> 2010a
<i>Potamogeton</i> hybrid	<i>P. gramineus</i>	<i>P. distinctus</i>	Du <i>et al.</i> 2010a
<i>Potamogeton</i> hybrid****	<i>P. gemmiparus</i>	<i>P. vaseyi</i>	Les <i>et al.</i> 2009
<i>Potamogeton</i> hybrid*	<i>P. pusillus</i> *	<i>P. berchtoldii</i> *	Du <i>et al.</i> 2010b*
<i>Potamogeton</i> hybrid	<i>P. foliosus</i>	<i>P. octandrus</i>	Du <i>et al.</i> 2010b
<i>Potamogeton</i> hybrid*	<i>P. distinctus</i> *	<i>P. nodosus</i> *	Du <i>et al.</i> 2010a*

\* No maternal parents were identified.

\*\* Named after morphological examination.

\*\*\* Suspected as within *P. pusillus* variation.\*\*\*\* Suspected as within *P. berchtoldii* variation.

Wang *et al.*'s (2007) '*Potamogeton* sp. hybrid,' displayed ITS polymorphism. Information on morphological intermediacy between their putative parental species and pollen sterility, which

could provide additional support for their hybrid origin, was not presented.

Zhang *et al.* (2008) sampled nearly the same taxa as Wang *et al.* (2007) in their phylogenetic

analyses of *trnT-trnF* DNA sequences (chloroplast genome), although Wang *et al.*'s (2007) '*Potamogeton* sp. hybrid' was not included. The phylogenetic trees that resulted from their study, however, display incongruence with respect to the phylogenetic position of several of the taxa that they included in their study. For example, Zhang *et al.*'s (2008) accession of *P. lucens* is most closely related to *P. wrightii* Morong in their study, but accessions of *P. lucens*, and its synonym, *P. dentatus* Hagstr., are most closely related to *P. gramineus* L. and only distantly related to *P. wrightii* in the chloroplast phylogenies published by others (Iida *et al.* 2004, Kaplan & Fehrer 2011, Du *et al.* 2011). This incongruence might be an additional source of information about the existence of *Potamogeton* hybrids and their origin.

The aim of our study was to use phylogenetic analyses of *trnT-trnF* and ITS sequence data to confirm the hybrid status and to infer the origin of the taxa that were labeled *P. lucens*, *P. natans*, and '*Potamogeton* sp. hybrid' in the studies of Wang *et al.* (2007) and Zhang *et al.* (2008). To achieve our goals, the sampling of Wang *et al.* (2007) and Zhang *et al.* (2008) with respect to these taxa was expanded with additional accessions and additional species. In addition, the latest monograph of *Potamogeton* (Wiegleb & Kaplan 1998) was used to select putative parental species of *P. lucens*, *P. natans*, and '*Potamogeton* sp. hybrid' on the basis of morphological similarities.

## Materials and methods

The *trnT-trnF* sequences of *Potamogeton* sensu stricto, or Clade I of Zhang *et al.* (2008), were obtained from GenBank, including the accessions of *P. lucens* and *P. natans*. '*Potamogeton* sp. hybrid' was not included in the study by Zhang *et al.* (2008) and *trnT-trnF* sequence data were therefore not available to us. The accessions of *P. alpinus* Balb. and *P. ×orientalis* Hagstr. of Zhang *et al.* (2008), both of which are distantly related to the putative hybrids we focused on, were omitted because the ITS sequence data were

unavailable for these taxa (Wang *et al.* 2007). Also the ITS sequences of *Potamogeton* sensu stricto, or Clade I of Wang *et al.* (2007), including the accessions of *P. lucens*, *P. natans*, and '*Potamogeton* sp. hybrid' were obtained from GenBank. Accessions of the hybrid species *P. hubeiensis* and *P. intortusifolius*, which were discussed in detail by Wang *et al.* 2007, were not included in the present study. Also the GenBank ITS accessions of *P. obtusifolius* Mert. & W. D. J. Koch were omitted, because of unavailability of *trnT-trnF* sequence data for the same specimen. However, because the results of Wang *et al.* (2007) indicated that *P. obtusifolius* is closely related to one of the putative hybrids, a different specimen of this taxon was sequenced (see below; Appendix 1). One of three ITS sequences of *P. lucens* from Wang *et al.* (2007) (DQ840265) was also excluded; it seemed to be a chimeric sequence resulting from PCR-mediated recombination between DQ840264 and DQ840266 (data not shown). On the basis of the phylogenetic relationships inferred by Wang *et al.* (2007) and Zhang *et al.* (2008), the following taxa were newly sequenced and included in our data sets to represent hybrids and potential parental species of hybrids: *P. ×inbaensis* (Chiba, Japan), *P. lucens* (Chiba, Japan), and *P. wrightii* (Chiba, Japan) from Ito *et al.* (2007) for comparison with the accessions of *P. lucens* of Wang *et al.* (2007) and Zhang *et al.* (2008); *P. distinctus* A. Benn. (Ibaraki, Japan), *P. obtusifolius* (Vladivostok, Russia), *P. octandrus* Poir. (Aomori, Japan), and *P. natans* (Hokkaido, Japan) for comparison with the accessions of *P. natans* of Wang *et al.* (2007) and Zhang *et al.* (2008); *P. praelongus* Wulfen (Hokkaido, Japan) and *P. wrightii* (the same as above) for comparison with the accession of '*Potamogeton* sp. hybrid' of Wang *et al.* (2007). *Potamogeton perfoliatus* L. (Hokkaido, Japan) was also added to the ITS and plastid data sets because it was most closely related to *P. praelongus* in Wang *et al.* (2007) and Zhang *et al.* (2008), yet only distantly related to *P. praelongus* in other studies (Iida *et al.* 2004, Kaplan & Fehrer 2011). Identifications of all newly sequenced plants were confirmed using Wiegleb & Kaplan (1998). The sequences

from our study have been registered with the DNA Data Bank of Japan (DDBJ); accession numbers are given in Appendix 1. Following Wang *et al.* (2007) and Zhang *et al.* (2008), *Stuckenia pectinata* (L.) Börner was used as the out-group.

The targeted regions of the newly sequenced specimens, as well as those previously sequenced by Ito *et al.* (2007), were amplified using the following primers: either “a” or Po-trnT2F (5′ – GCATAGGAACTTAATAAACCCCA – 3′) and “b” for the chloroplast *trnT*–*trnL* (787–860 bp or 745–785 bp, respectively), “c” and “d” for *trnL* intron (591 or 593 bp), and “e” and “f” for *trnL*–*trnF* (403–407 bp) (Taberlet *et al.* 1991) and ITS-4 and ITS-5 for the ITS region (697–715 bp; Baldwin 1992). PCR amplification was performed following the procedure of Ito *et al.* (2007). For *P. ×inbaensis*, subsequent cloning of 16 clones was carried out to identify potential ITS polymorphism (Ito *et al.* 2007). Electropherograms were examined by eye using the Genetyx-Win Version 3 software (Software Development Co. Ltd., Tokyo, Japan).

Sequences of *trnT*–*trnF* and ITS were manually aligned and the simple indel coding of Simmons & Ochoterena (2000) was used to code gaps. However, gaps associated with mononucleotide repeats were removed from the phylogenetic analyses, because homology assessment can be very difficult for these repeated nucleotides (Kelchner 2000) and they might be technical artifacts of the PCR amplification (Clarke *et al.* 2001). Phylogenetic analyses were independently performed for the ITS and *trnT*–*trnF* data sets, because we detected incongruence between these DNA regions (see below). One representative sequence was used in the phylogenetic analyses for taxa for which different specimens yielded identical sequences. Phylogenetic inference was performed using maximum parsimony (MP) in PAUP\* and Bayesian inference (BI; Yang & Rannala 1997) in MrBayes 3.1.2. (Ronquist & Huelsenbeck 2003). In the MP analysis, a heuristic search was performed with 100 random addition sequence replicates involving tree-bisection-reconnection (TBR) branch swapping, with the

Mul-trees option in effect. The MaxTree option was set at no limits for the analysis. Bootstrap analyses (Felsenstein 1985) were performed using 1,000 replicates with TBR branch swapping and a simple addition sequence. In the BI analysis, hierarchical likelihood ratio tests implemented in the program MrModeltest 3.7 (Nylander 2002) were used for substitution model selection (GTR+G and HKY+G for *trnT*–*trnF* and ITS). For gap characters, the datatype = standard option of MrBayes was used. The Bayesian Markov Chain Monte Carlo algorithm was run for 400,000 and 800,000 generations in the *trnT*–*trnF* and ITS analyses until the average standard deviation of split frequency dropped below 0.01. Four incrementally heated chains starting from random trees were used and these were sampled every 100 generations. The data from the first 25% of the total number of generations (100,000 and 200,000 generations for *trnT*–*trnF* and ITS data set, respectively) were discarded as burn-in, and the remaining trees were used to calculate a 50% majority-rule consensus tree and to determine posterior probabilities for clades. The data matrices and the MP trees are available from the TreeBASE database (<http://www.treebase.org>, study accession S13337).

The *Potamogeton perfoliatus*-related ITS sequences of ‘*Potamogeton* sp. hybrid’ was compared with those of closely related accessions, i.e., *P. perfoliatus* and *P. praelongus* of Wang *et al.* (2007), *P. perfoliatus* in the present study (Hokkaido, Japan), and *P. ×anguillanus* of Du *et al.* (2009). Accessions of *P. intortusifolius* of Wang *et al.* (2007) were omitted from this comparison, because their two ITS sequences represented either *P. wrightii* (DQ840293) or a chimeric sequence resulting from recombination between *P. wrightii* and *P. perfoliatus* ITS copies (DQ840292).

## Results

### Phylogenetic analysis of *trnT*–*trnF*

The length of *trnT*–*trnF* alignment containing 33 accessions totaled 2028 bp, of which 104 characters were parsimony-informative. Twenty five

equally parsimonious MP trees (tree length = 345, consistency index = 0.87, retention index = 0.90) were obtained. Because MP and BI analyses recovered largely congruent topologies, only one of most parsimonious MP trees is shown (Fig. 1). Although the basal parts of the *trnT-trnF* tree were poorly supported, several clades were obtained that were well supported in either or both

the MP and BI analyses; those were tTF-I with *P. distinctus*, *P. lucens* (China), *P. malainoides*, *P. perfoliatus*, *P. praelongus* (China), and *P. wrightii* (99% MP bootstrap support [BS] and 0.64 Bayesian posterior probability [PP]), tTF-II with *P. lucens* (Japan) and *P. gramineus* (98% BS and 1.00 PP), tTF-III with *P. crispus* and *P. maackianus* (94% BS and 1.00 PP), tTF-IV with *P. natans* and

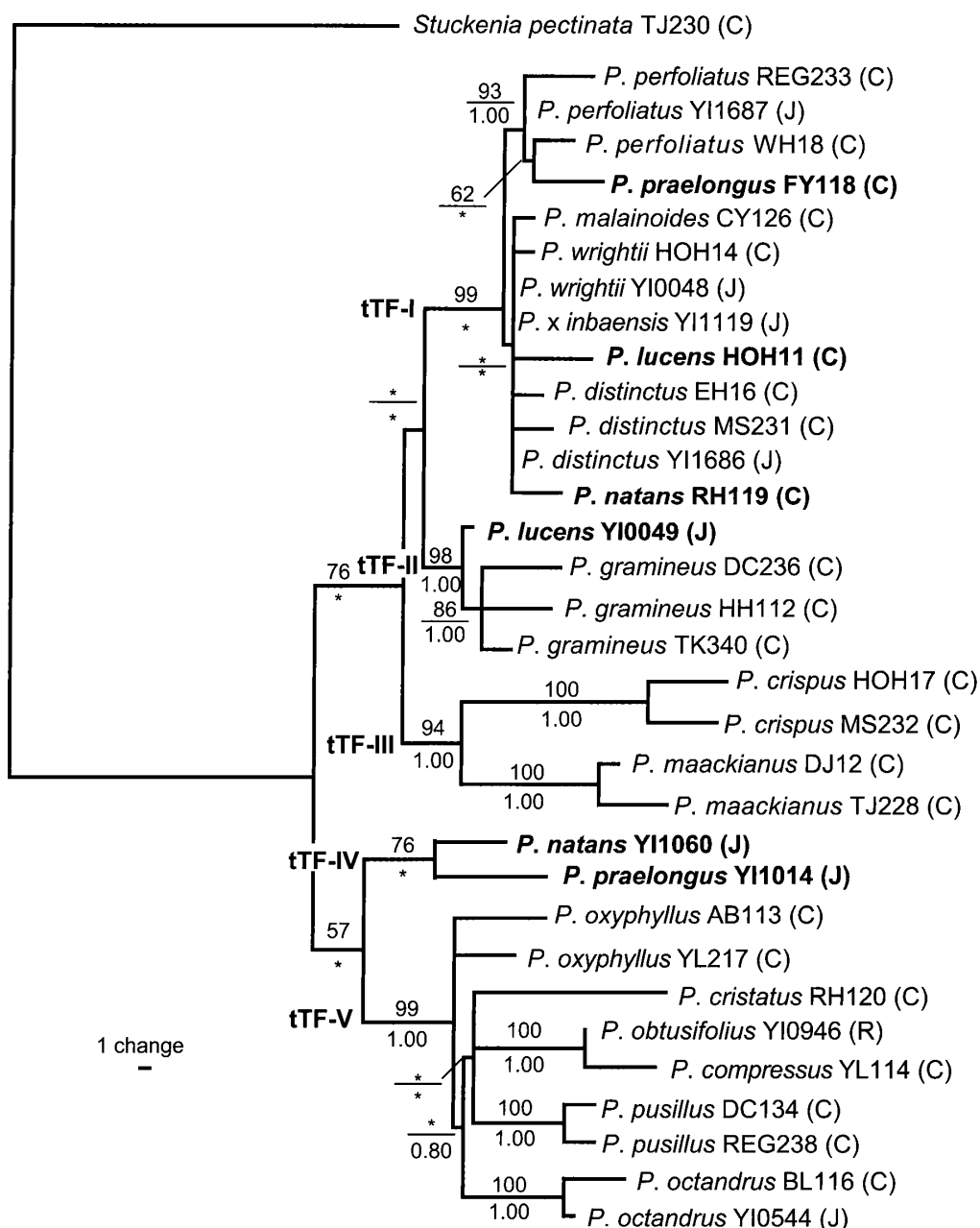


FIG. 1. One of 25 most parsimonious trees of *Potamogeton* sensu stricto based on plastid *trnT-trnF* sequences with *Stuckenia pectinata* as outgroup. ACCTRAN optimization was used for branch length measures of the phylogram. Numbers above the branches indicate bootstrap support (BS) calculated in maximum parsimony analysis and those below indicate Bayesian prior probabilities (PP). BP < 50% and PP < 0.70 are indicated by asterisks. The species placed in polyphyletic clades are in bold face (*P. lucens*, *P. natans*, and *P. praelongus*). The origin of each accession is shown after voucher information as C (China), J (Japan), and R (Russia).

*P. praelongus* (Japan) (76% BS and <0.50 PP), and tTF-V with *P. cristatus*, *P. compressus*, *P. octandrus*, *P. oxyphyllus*, *P. pusillus* (99% BS and 1.00 PP). The results indicate that *P. lucens* (*P. lucens* HOH11 [China] and *P. lucens* YI0049 [Japan]), *P. natans* (*P. natans* RH119 [China] and *P. natans* YI1060 [Japan]), and *P. praelongus* (*P. praelongus* FY118 [China] and *P. praelongus* YI1014 [Japan]) are polyphyletic.

#### Phylogenetic analysis of ITS

The length of the ITS alignment composed of 60 accessions totaled 734 bp, of which 100 characters were parsimony-informative. Eight MP trees (tree length = 305, consistency index = 0.84, retention index = 0.92) were obtained and because MP and BI analyses recovered largely congruent topologies, only one of the most parsimonious MP trees is shown (Fig. 2). Again, several mostly well supported major clades were obtained: ITS-I with *P. distinctus*, *P. malainoides*, *P. natans* (China), *P. obtusifolius* (China), *P. perfoliatus*, *P. praelongus* (China), and *P. wrightii* (98% BS and 1.00 PP) and ITS-V with *P. gramineus*, *P. lucens* (Japan), *P. natans* (Japan), and *P. praelongus* (Japan) (87% BS and 1.00 PP). The former clade consisted of two subclades, ITS-I\_1 with *P. perfoliatus* and *P. praelongus* (China) (90% BS and 1.00 PP), and ITS-I\_2 with *P. distinctus*, *P. malainoides*, *P. natans* (China), *P. obtusifolius* (China), and *P. wrightii* (99% BS and 1.00 PP). The latter clade included two subclades, ITS-V\_1 with *P. natans* (Japan) and *P. praelongus* (Japan) (98% BS and 1.00 PP) and ITS-V\_2 with *P. gramineus* and *P. lucens* (China, Japan) (95% BS and 1.00 PP). The other clades are ITS-II with *P. compressus*, *P. obtusifolius* (Russia), and *P. oxyphyllus*, which is only poorly supported (58% BS and 0.92 PP), ITS-III with two accessions of *P. cristatus* (100% BS and 1.00 PP), the poorly supported ITS-IV clade containing *P. octandrus* and *P. pusillus* (64% BS and 0.60 PP), and ITS-VI with *P. crispus* and *P. maackianus* (66% BS and 0.99 PP). The divergent ITS copies of *P. lucens*, *P. natans*, and ‘*Potamogeton* sp. hybrid’ of Wang *et al.* (2007) were placed in distantly related clades: ITS-I and ITS-V, ITS-I and ITS-

IV, and ITS-I\_1 and ITS-I\_2, respectively. In addition to these three taxa, *P. obtusifolius* (*P. obtusifolius* Dongbei 03 [China] and *P. obtusifolius* YI0946 [Japan]) and *P. praelongus* (*P. praelongus* FY118 [China] and *P. praelongus* YI1014 [Japan]) were resolved as polyphyletic. None of the divergent ITS copies of *P. natans* RH119 (China) is resolved as closely related to the ITS accession of *P. natans* YI1060 (Japan). *Potamogeton lucens* specimen HOH11 (China) contained divergent ITS sequences, whereas only identical ITS copies were obtained from *P. lucens* YI0049 (Japan). *Potamogeton perfoliatus* exhibited relatively high genetic variation compared to other species for which multiple accessions were included, e.g., *P. distinctus* and *P. gramineus* (Fig. 2).

#### Comparison of trnT–trnF and ITS phylogenies

A comparison of the *trnT*–*trnF* and ITS *Potamogeton* phylogenies recovered several largely congruent clades, i.e., tTF-I and ITS-I, tTF-II and ITS-V\_2, tRF-III and ITS-VI, tTF-IV and ITS-V\_1, and tTF-V and ITS-II, ITS-III, and ITS-IV. Several incongruent clades were also identified, however, e.g., the monophyletic tTF-V clade was resolved as paraphyletic in the ITS cladograms (ITS-II, ITS-III, and ITS-IV; Fig. 3), as well as individual accessions that obtained different and conflicting phylogenetic positions in the *trnT*–*trnF* and ITS trees.

#### A further comparison of *P. perfoliatus*-related ITS sequences

A multiple sequence alignment was performed using sequences of clade ITS-I\_1 (Fig. 2). This alignment shows two main groups: one contains the *P. perfoliatus* accessions of Wang *et al.* (2007) and Du *et al.* (2009) and *P. ×anguillanus* and the other is composed of the *P. perfoliatus* sequence generated for the present study, *P. praelongus*, and ‘*Potamogeton* sp. hybrid’ of Wang *et al.* (2007) (Table 2).

## Discussion

Molecular phylogenetic analyses were employed to verify the hybrid status and to infer the

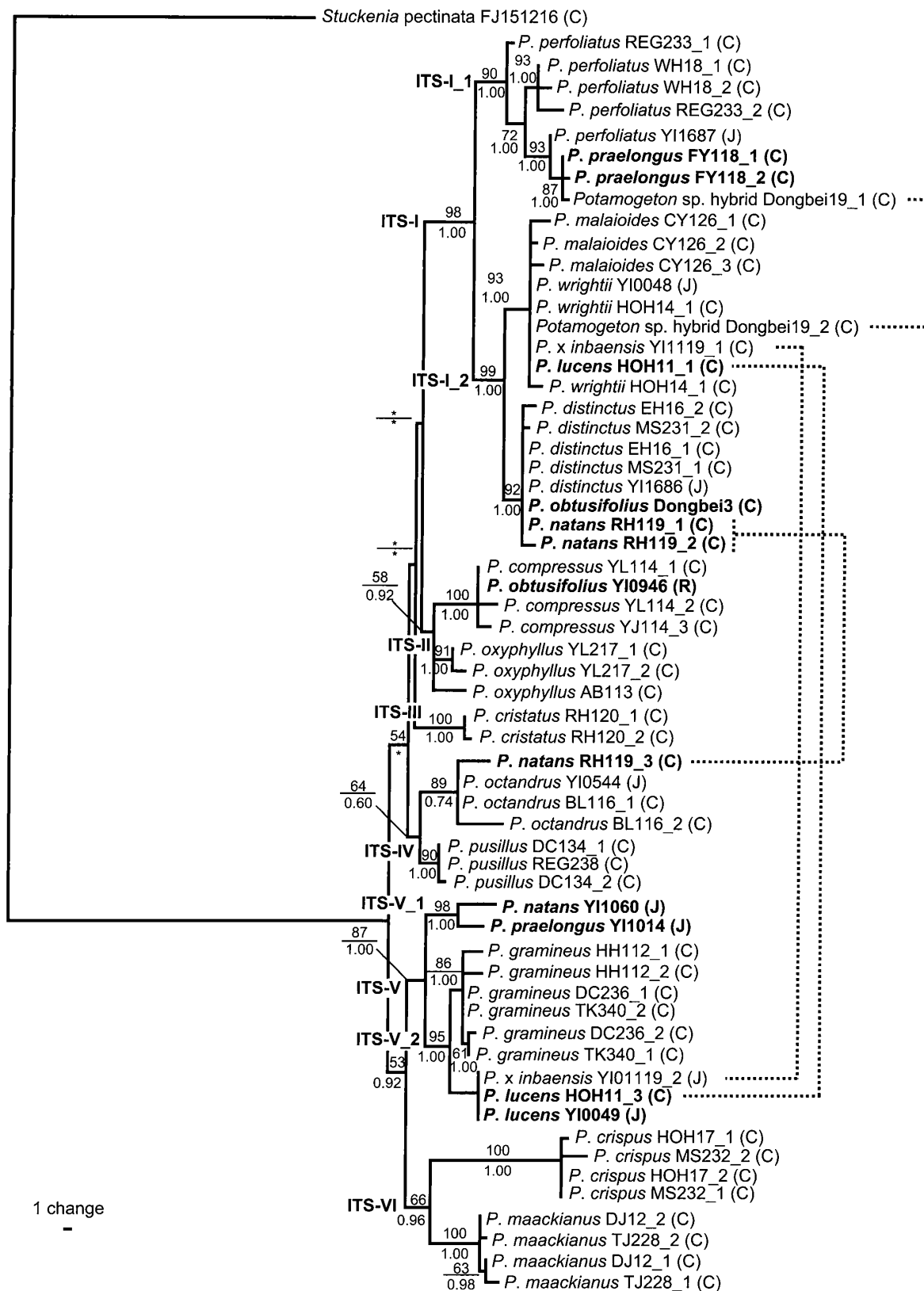


FIG. 2. One of eight most parsimonious trees of *Potamogeton* sensu stricto based on nuclear ITS sequences with *Stuckenia pectinata* as outgroup. ACCTRAN optimization was used for branch length measures of the phylogram. Numbers above the branches indicate bootstrap support (BS) calculated in maximum parsimony analysis and those below indicate Bayesian prior probabilities (PP). BP <50% and PP <0.70 are indicated by asterisks. The accessions which have far related heterogeneous ITS sequences are found; each sequence was connected by dotted lines. The species placed in polyphyletic clades are in bold face (*P. lucens*, *P. natans*, *P. obtusifolius*, and *P. praelongus*). The origin of each accession is shown after voucher information as C (China), J (Japan), and R (Russia).

TABLE 2. Comparison of the *P. perfoliatus*-related ITS sequence of *Potamogeton* sp. hybrid of Wang *et al.* (2007) and its related species, including *P. ×anguillanus* of Du *et al.* (2009) deposited in GenBank.

Locality	Reference*	GenBank accession	69	146	175	220	235	283	317	422	423	514	564	571	604	618	623	647-649
<i>Potamogeton ×anguillanus</i> **																		
China (Shanxi)	2	-	-	T	G	G	A	A	A	A	T	T	C	A	A	G	T	CAA
China (Hubei)	2	-	-	T	G	G	A	A	A	A	T	T	C	A	A	G	T	CAA
<i>Potamogeton perfoliatus</i>																		
China (Hubei)	1	DQ840288	-	T	G	G	A	A	A	A	T	T	C	A	A	G	T	CAA
		DQ840289	-	T	G	G	G	A	A	A	T	C	C	A	A	G	T	CAA
China (Sichuan)	1	DQ840290	-	T	G	G	A	G	A	A	C	T	C	A	A	A	T	CAA
		DQ840291	-	T	G	G	A	A	A	T	T	T	C	G	G	G	C	CAA
China (Shanxi)	2	EU741047	-	T	G	G	A	A	A	A	T	T	C	A	A	G	T	CAA
China (Heilongjiang)	2	EU741048	-	T	G	G	A	A	A	A	T	T	C	A	A	G	T	CAA
Japan (Hokkaido)***	3	AB744008	-	C	T	G	A	A	A	A	T	T	T	A	C	G	C	CAA
<i>Potamogeton praelongus</i>																		
China (Heilongjiang)	1	DQ840318	-	C	T	T	A	A	A	A	T	T	T	A	C	G	C	---
	1	DQ840319	T	C	T	T	A	A	A	A	T	T	T	A	C	G	C	---
<i>Potamogeton</i> sp. hybrid																		
China (Heilongjiang)	1	DQ840325	-	C	T	T	A	A	G	A	T	T	T	A	C	G	C	---

\* 1. Wang *et al.* (2007), 2. Du *et al.* (2009), 3. Present study  
\*\* Cited under the name of *P. intortusifolius* in Du *et al.* (2009).  
\*\*\* Cultivating collections at the Tsukuba Botanical Garden.

origin of three putative hybrids *P. lucens*, *P. nantans*, and ‘*Potamogeton* sp. hybrid’ included in the studies of Wang *et al.* (2007) and Zhang *et al.* (2008). Our analyses revealed that these studies included three unrecognized *Potamogeton* hybrids because heterogeneous ITS sequences of three accessions were placed in distantly related clades. Furthermore, maternally inherited plastid DNA sequence data allowed inference of the origin of two out of the three cases of hybridization. In the following paragraphs, accessions for which identifications need to be revised are shown with single quotation marks, e.g., ‘*P. lucens*’ and ‘*P. malainoides*’.

The *trnT*–*trnF* data used in the present study shows that the plastid accession that was labeled ‘*P. lucens*’ (HOH11 [China]) in the study of Zhang *et al.* (2008) is most closely related to *trnT*–*trnF* accessions of *P. distinctus*, *P. wrightii* (including ‘*P. malainoides*’ CY126 [China]) and *P. ×inbaensis*. A second specimen of *P. lucens* (YI0049 [Japan]), however, appears to be most similar to *P. gramineus* in the *trnT*–*trnF* cladograms (Fig. 1). A similar conflict in the phylogenetic placement of different accessions of *P. lucens* was observed in the ITS phylogenies. Wang *et al.* (2007) retrieved two divergent ITS sequences from a specimen identified as ‘*P. Lucens*’ (HOH11) and which was also used in the study by Zhang *et al.* (2008). One of the ITS copies was resolved in a similar phylogenetic position as the *trnT*–*trnF* accession of this plant, whereas the other ITS copy was most closely related to an accession of *P. lucens* from a different specimen (YI0049) and one of the two divergent copies of *P. ×inbaensis*. The latter species was first recorded in Japan and suggested to be of hybrid origin based on morphological intermediacy between its putative parental species *P. lucens* and *P. wrightii*, which occasionally co-occur, and pollen sterility (Kadono 1983). Its hybrid status and parents were later confirmed genetically with the identification of *P. wrightii* as the maternal parent (Ito *et al.* 2007) and vice versa (Amano *et al.* 2008). The similar phylogenetic placement of divergent ITS copies of specimen HOH11 and those of *P. ×inbaensis* and the alternative phylogenetic

positions of accessions of this specimen with that of *P. lucens* (YI0049) strongly suggests that specimen HOH11 is *P. ×inbaensis* instead of *P. lucens*. Geographical data show that this conclusion is certainly plausible; the inferred maternal parent of HOH11, *P. wrightii*, grows at the same locality and the second parent, *P. lucens*, occurs in the same province (Guo *et al.* 2010). The incorrect identification by Wang *et al.* (2007) and Zhang *et al.* (2008) of this hybrid is not unexpected, since similar difficulties in identifying hybrids and their parental species in *Potamogeton* was reported by Ito *et al.* (2007).

The ‘*Potamogeton* sp. hybrid’ specimen of Wang *et al.* (2007) also contained divergent ITS copies that were resolved in different phylogenetic positions, thereby confirming this specimen to be of hybrid origin. One of the ITS copies was positioned in a clade of *P. perfoliatus* (including ‘*P. praelongus*’ FY118 [China]) specimens, whereas the other ITS copy suggested genetic affinities with *P. wrightii* (including ‘*P. malainoides*’ CY126 [China]). Because of the absence of *trnT*–*trnF* sequences of the ‘*Potamogeton* sp. hybrid’ specimen (hereafter called “Hybrid\_α”), it is presently not possible to determine which species is the probable maternal parent. A detailed study of the ITS sequences of this hybrid and its putative parents revealed that the *P. perfoliatus*-related genome of “Hybrid\_α” is slightly different from that of *P. ×anguillanus* Koidz., the confirmed hybrid between *P. perfoliatus* and *P. wrightii* (Koidzumi 1929, Du *et al.* 2009; Table 2), indicating that a different species than *P. perfoliatus*, although closely related and perhaps cryptic, has been involved in the hybridization event. Neither *P. perfoliatus* sensu lato nor *P. wrightii* was observed growing with “Hybrid\_α” (Wang *et al.* 2007), yet *P. ×anguillanus* with a *P. wrightii*-related chloroplast genome has been collected at the same locality (Zhang *et al.* 2008). Both parental species, *P. perfoliatus* s.l. and *P. wrightii*, however, occur in the same province (Guo *et al.* 2010) and this makes hybridization between them not impossible. “Hybrid\_α” is therefore most likely a hybrid between either *P. perfoliatus* s.l. (or an undescribed cryptic species of *P.*

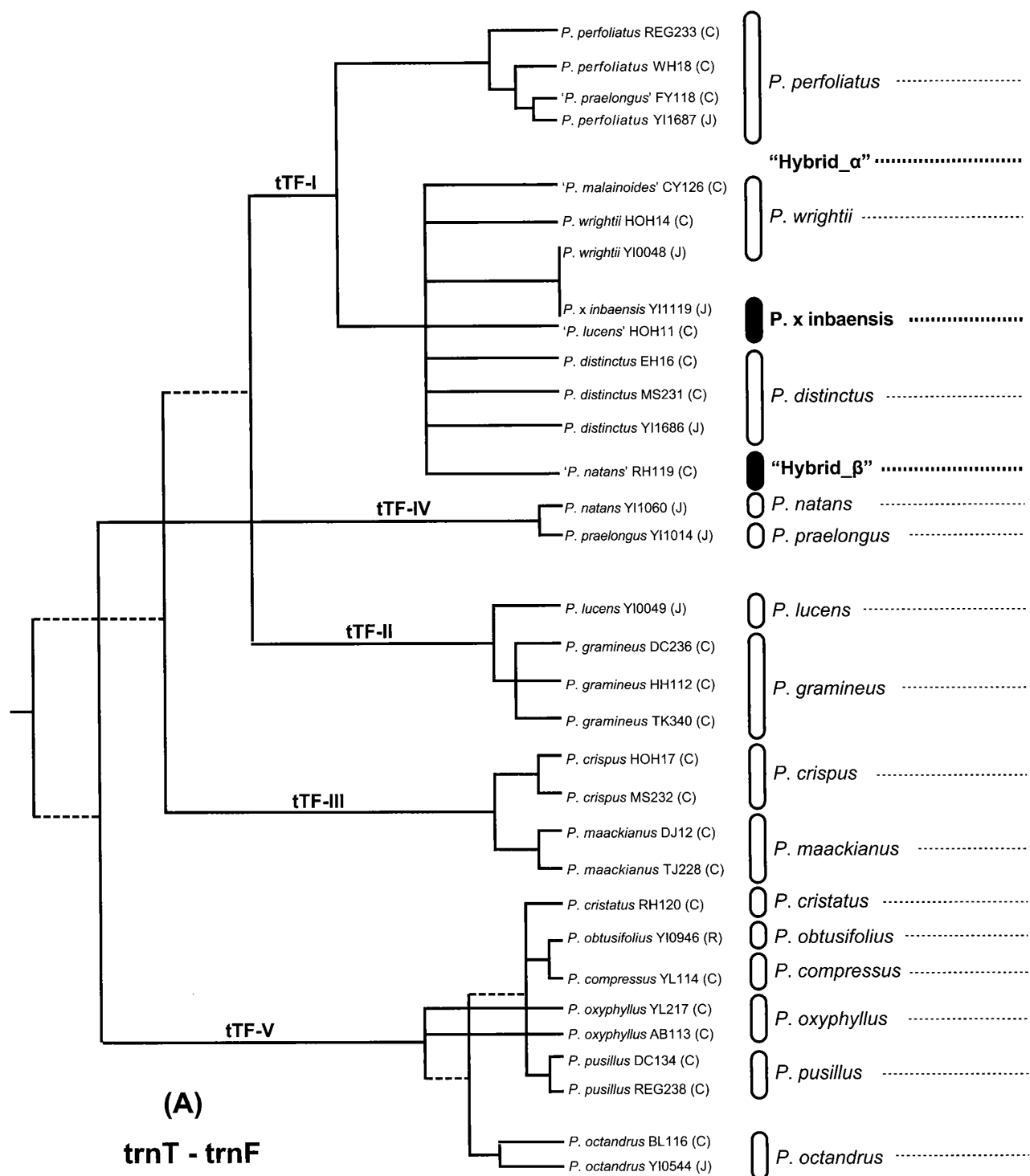
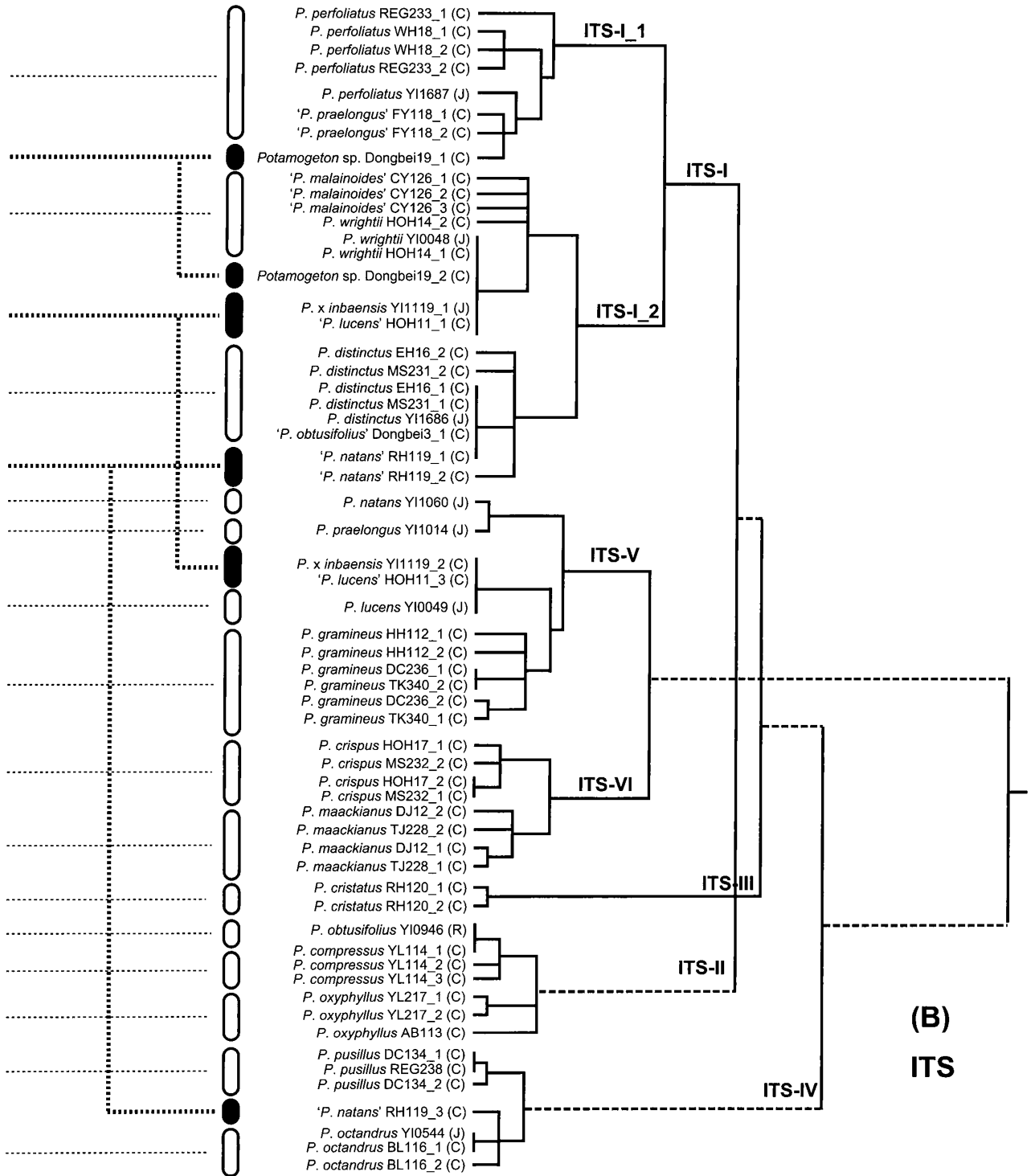


Fig. 3. Phylogenetic relationships of *Potamogeton* sensu stricto inferred from (A) *trnT-trnF* and (B) ITS trees. The outgroup are trimmed to clarify ingroup phylogeny. Some less resolved *trnT-trnF* and ITS clades are modified to fit each other. Dotted lines connect corresponding clades between *trnT-trnF* and ITS trees. Three cases of hybridization are in bold face (*P. x inbaensis*, Hybrid\_α, and Hybrid\_β). The origin of each accession is shown after voucher information as C (China), J (Japan), and R (Russia). Some accessions requiring revision of the identifications are shown within single quotation marks, e.g., '*P. lucens*'; they are reclassified into the latest monograph's systematics (Wiegand & Kaplan 1998).



*perfoliatus*) and *P. wrightii*.

Thus far, *Potamogeton* hybrids have only been reported between either broad leaved species or linear leaved species; in other words, within tetraploids or diploids (e.g., Du *et al.* 2010a, 2010b). Here we report the only known exception to this pattern. The specimen labeled '*P. natans*' (RH119 [China]) in Wang *et al.* (2007) and Zhang *et al.* (2008) yielded an *trnT-trnF* sequence that is most closely related to *P. distinctus* and *P. wrightii* (including '*P. malaionoides*' CY126) and divergent ITS copies of which two were resolved as being most closely related to *P. distinctus* (including '*P. obtusifolius*' Dongbei3\_1 [China]) and one to *P. octandrus* (Fig. 3). These findings suggest that specimen RH119 is a hybrid between the tetraploid species *P. distinctus* and diploid *P. octandrus* ("Hybrid $\beta$ "), the former being the maternal and the latter the paternal parent. Both parental species occur in the same province (Guo *et al.* 2010).

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APPENDIX 1. List of the GenBank accessions of *Potamogeton* species and its outgroup, *Stuckenia*, used in the phylogenetic analyses on *trnT-trnF* (*trnT-trnL*, *trnL* intron, and *trnL-trnF*) and ITS. Sequences determined in the present study are shown in underline and their voucher information is provided. Accessions obtained from Wang *et al.* (2007) and Zhang *et al.* (2008) are numbered; those are added after each species name.

Accession	<i>trnT-trnL</i>	<i>trnL</i>	<i>trnL-trnF</i>	ITS	Locality	Voucher
<i>P. compressus</i> _1	EF471054	EF428389	EF432079	DQ840310 DQ840311 DQ840312	China	YL114 (WH)
<i>P. crispus</i> _1	EF471048	EF428382	EF432072	DQ840284 DQ840285	China	HOH17 (WH)
<i>P. crispus</i> _2	EF471094	EF428404	EF432094	DQ840286 DQ840287	China	MS232 (WH)
<i>P. cristatus</i> _1	EF471059	EF428394	EF432084	DQ840323 DQ840324	China	RH120 (WH)
<i>P. distinctus</i>	<u>AB744025</u>	<u>AB744013</u>	<u>AB744019</u>	<u>AB744007</u>	Japan (Ibaraki)*	YI1686 (TNS)
<i>P. distinctus</i> _1	EF471047	EF428381	EF432071	DQ840280 DQ840281	China	EH16 (WH)
<i>P. distinctus</i> _2	EF471068	EF428403	EF432093	DQ840282 DQ840283	China	MS231 (WH)
<i>P. gramineus</i> _1	EF471052	EF428387	EF432077	DQ840301 DQ840302	China	HH112 (WH)
<i>P. gramineus</i> _2	EF471073	EF428408	EF432098	DQ840303 DQ840304	China	DC236 (WH)
<i>P. gramineus</i> _3	EF471075	EF428410	EF432100	DQ840305 DQ840306	China	TK340 (WH)
<i>P. ×inbaensis</i>	<u>AB695138</u>	<u>AB695128</u>	<u>AB695134</u>	AB206990 AB206991	Japan (Chiba)	YI1119 (TNS) (TNS9525987)
<i>P. lucens</i>	<u>AB695137</u>	<u>AB695129</u>	<u>AB695133</u>	AB206990	Japan (Chiba)	YI0049 (TNS) (TNS9525970)
<i>P. lucens</i> _1	EF471043	EF428376	EF432066	DQ840264 DQ840265 DQ840266	China	HOH11 (WH)
<i>P. maackianus</i> _1	EF471044	EF428377	EF432067	DQ840267 DQ840268	China	DJ12 (WH)
<i>P. maackianus</i> _2	EF471066	EF428401	EF432091	DQ840269 DQ840270 DQ840271	China	TJ228 (WH)
<i>P. malainoides</i> _1	EF471065	EF428400	EF432090	DQ840338 DQ840339 DQ840340	China	CY126 (WH)
<i>P. natans</i>	<u>AB744024</u>	<u>AB744012</u>	<u>AB744018</u>	<u>AB744006</u>	Japan (Hokkaido)	YI1060 (TNS)
<i>P. natans</i> _1	EF471058	EF428393	EF432083	DQ840320 DQ840321 DQ840322	China	RH119 (WH)
<i>P. obtusifolius</i>	<u>AB744022</u>	<u>AB744010</u>	<u>AB744016</u>	<u>AB744004</u>	Russia (Vladivostok)	YI0946 (TNS)
<i>P. octandrus</i>	<u>AB744021</u>	<u>AB744009</u>	<u>AB744015</u>	<u>AB744003</u>	Japan (Aomori)	YI0544 (TNS)
<i>P. octandrus</i> _1	EF471055	EF428390	EF432080	DQ840316 DQ840317	China	BL116 (WH)
<i>P. oxyphyllus</i> _1	EF471053	EF428388	EF432078	DQ840307	China	AB113 (WH)
<i>P. oxyphyllus</i> _2	EF471056	EF428391	EF432081	DQ840308 DQ840309	China	YL217 (WH)
<i>P. perfoliatus</i>	<u>AB744026</u>	<u>AB744014</u>	<u>AB744020</u>	<u>AB744008</u>	Japan (Hokkaido)*	YI1687 (TNS)
<i>P. perfoliatus</i> _1	EF471049	EF428383	EF432073	DQ840288 DQ840289	China	WH18 (WH)
<i>P. perfoliatus</i> _2	EF471070	EF428405	EF432095	DQ840290 DQ840291	China	REG233 (WH)
<i>P. praelongus</i>	<u>AB744023</u>	<u>AB744011</u>	<u>AB744017</u>	<u>AB744005</u>	Japan (Hokkaido)	YI1014 (TNS)
<i>P. praelongus</i> _1	EF471057	EF428392	EF432082	DQ840318 DQ840319	China	FY118 (WH)
<i>P. pusillus</i> _1	EF471071	EF428406	EF432096	DQ840294 DQ840295	China	DC134 (WH)
<i>P. pusillus</i> _2	EF471074	EF428409	EF432099	DQ840296	China	REG238 (WH)
<i>P. wrightii</i>	<u>AB695139</u>	<u>AB695131</u>	<u>AB695135</u>	AB206991	Japan (Chiba)	YI0048 (TNS) (TNS9525993)
<i>P. wrightii</i> _1	EF471045	EF428379	EF432069	DQ840274 DQ840275	China	HOH14 (WH)
<i>S. pectinata</i> _1	EF471067	EF428402	EF432092	DQ840279	China	TJ230 (WH)

\* Cultivating collections at Tsukuba Botanical Garden